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A chitin-oligomer binding peptide obtained by screening of a phage display random peptide library and its affinity modulation corresponding to oxidation–reduction state

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Abstract

Screening of a phage display random 9-mer peptide library, in which cysteine residues were at the both terminals of the 7-mer random region, was performed to obtain an oligopeptide that recognizes a chitin-oligomer. Affinity of the obtained peptide (Cys-Ser-Arg-Thr-Thr-Arg-Thr-Arg-Cys) to chitotriose was modulated by its oxidation–reduction state. Only the oxidized form exhibited specific binding to the target molecule, chitotriose. This is the first report of reversible affinity modulation of a synthetic oligopeptide which can recognize a neutral saccharide.

Keywords: Chitin-oligomer; Oxidation–reduction; Peptide library

1. Introduction

Functional elucidation of oligosaccharides in organisms is one of the most important subjects in the post-genome researches. Ligands that can recognize the structures of arbitrary sequences of oligosaccharides are very useful for such research. In particular, short peptides are one of the most suitable materials for molecular recognition because of their superior biocompatibility. However, obtaining ligands for neutral saccharides is thought to be difficult because the main interaction between peptides and saccharides is not a full-charge electrostatic interaction. The likely interaction mode in aqueous solution is only a hydrogen bonding that individually is thought not to lead to strong interaction. Such weak binding is not enough to be selected as positive clone through a biopanning screening. Screening a combinatorial library should be one possible answer to the problem of selecting ligands that bind their targets by 'weak' hydrogen bonds. Here we tried to obtain peptide ligands that recognize a neutral saccharide by a combinatorial bioengineering technique using a phage display system. The phage display method is one of the most important combinatorial bioengineering techniques by which arbitrary random peptides can be displayed on the bacteriophage coat proteins. An *E. coli* filamentous phage is used most often as a scaffold. Its library size should surpass 10^{10} [1,2]. To date, phage display library screening has afforded several specific peptide ligands that bind antibodies, enzymes, and receptors [3–6]. Small molecules, such as biotin have also been targeted by phage display library screening [7]. However, only few reports on a phage display peptide library screening against neutral saccharide have been made due to the difficulties mentioned above [8,9]. Recently, to overcome the present problem we developed a new screening protocol for phage display libraries, in which media including organic solvent were employed in order to emphasize the hydrogen bonding that is a main mode for recognizing saccharides [10]. Herein we report the screening of a phage display random peptide library, in which cysteine residues are linked to the both terminals of a random hepta-peptide region (-Cys-X-X-X-X-X-X-Cys-) to obtain an oligopeptide that recognizes a chitin-oligomer. The obtained peptide bound specificcally to chitotriose only in the oxidized state. This is the first report on reversible affinity modula-

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tion of a synthetic oligopeptide that can recognize neutral saccharides.

2. Results and discussion

2.1. Selection of a random peptide library for affinity to chitin-oligomer

The random peptide phage display library that we used is "pSKAN phage" which is derived from filamentous phage M13KO7 and displays a trypsin inhibitor containing structurally flexible random sequences of seven amino acids of which both terminals are held in the cysteine residue (-Cys-X-X-X-X-X-X-Cys-) on the N-terminal of the coat protein pIII. After five rounds of biopanning, we obtained fifteen peptide sequences as summarized in Table 1.

A remarkable tendency of specific amino acid appearance was observed even though the obtained peptides did not converge into certain consensus sequences. In the obtained sequences, serine, threonine and arginine, which can form effective hydrogen bonds to saccharides, tend to occur. The sequences could be categorized into four classes. The first group is the 'Thr/Ser-Arg-Thr/Ser group' (clones #1–3). The second group is the 'Thr/Ser-X-Ser group' (clones #4 and 5). The third group is the 'Arg-Thr/Ser group' (clones # 6 and 7). The fourth group consists of the others (clones # 8–15). Clones 1, 4 and 6 were chosen as representatives to be synthesized. The synthesized peptides were subjected to an affinity assay.

2.2. Affinity modulation of synthetic peptide for chitin binding corresponding to the oxidation–reduction state

The synthetic peptides were thought to represent different conformations corresponding to their oxidation–reduction status because they had cysteine residues at each terminal. Therefore, both an oxidized form and a reduced form were prepared for all peptides and were subjected to an assay to the saccharides. Results were summarized in Table 2.

Table 1
Sequences in random peptide region of selected phage clone after five repetitions of biopanning

Clone #	Sequence
1	Gly-Gly-Cys-**Ser-Arg-Thr-Thr-Arg-Thr-Arg**-Cys-Gly-Gly-
2	Gly-Gly-Cys-Gly-Asp-Ala-Phe-Ser-**Arg-Ser**-Cys-Gly-Gly-
3	Gly-Gly-Cys-**Ser-Arg-Thr-Arg**-Trp-**Thr**-Met-Cys-Gly-Gly-
4	Gly-Gly-Cys-Leu-**Thr**-Leu-**Ser**-Ala-Gly-Tyr-Cys-Gly-Gly-
5	Gly-Gly-Cys-Gly-Val-**Ser**-Val-**Ser**-Ala-Tyr-Cys-Gly-Gly-
6	Gly-Gly-Cys-Glu-Ala-**Arg-Ser**-Lys-Glu-Gly-Cys-Gly-Gly-
7	Gly-Gly-Cys-Val-Gly-**Arg-Thr**-Cys-**Thr-Arg**-Cys-Gly-Gly-
8	Gly-Gly-Cys-Leu-Asn-His-**Thr**-Glu-Cys-Lys-Cys-Gly-Gly-
9	Gly-Gly-Cys-Gly-His-**Arg**-Gly-Asp-Leu-Phe-Cys-Gly-Gly-
10	Gly-Gly-Cys-**Arg**-Val-Asn-Gly-**Arg**-Ala-**Arg**-Cys-Gly-Gly-
11	Gly-Gly-Cys-Thr-Ile-Leu-His-Pro-Met-**Ser**-Cys-Cys-Gly-
12	Gly-Gly-Cys-Leu-**Thr**-Leu-**Ser**-Ala-Gly-Tyr-Cys-Cys-Gly-
13	Gly-Gly-Cys-**Thr**-Lys-Ile-Tyr-Asp-Gly-Val-Cys-Gly-Gly-
14	Gly-Gly-Cys-**Ser**-Leu-His-Met-Ser-Lys-Lys-Cys-Gly-Gly-
15	Gly-Gly-Cys-Trp-**Ser**-Gly-Val-**Ser**-Leu-Cys-Cys-Gly-Gly-

Shaded regions indicate random peptides. Arg, Ser and Thr residues are underlined. The two boxed Cys residues are the fixed sequence.

Table 2
Binding assay of synthetic peptides

Peptide #	Subtraction value (angle) (10^{-3}°)	
	Chitotriose	Cellotriose
Clone 1 (oxidized)	5.6	0.9
Clone 1 (reduced)	0.3	1.3
Clone 4 (oxidized)	1.4	1.6
Clone 4 (reduced)	1.6	0.3
Clone 6 (oxidized)	0.9	0.6
Clone 6 (reduced)	0.7	0.8

Only oxidized clone 1 exhibited significant specific binding to chitotriose, although its reduced form did not. Clones 4 and 6 did not show any significant binding to the target (chitotriose) in either their oxidized or reduced forms. The oxidized form of clone 1 was immobilized onto an SPR sensor chip and subjected to a global fitting analysis for cellotriose and chitotriose. The binding parameters, such as dissociation constant (K_D) were calculated based on the sensorgram (Table 3). The K_D value for chitotriose was remarkably lower than that for cellotriose. The result strongly suggested that clone 1 peptide in oxidized form specifically recognized the chitin-oligomer. The linkage fashions of chitotriose and cellotriose are all beta-1,4 linkages. The structure of chitotriose is different from cellotriose only at 2′ position of the sugar. Accordingly, the methylcarbonylamino group at the 2′ position should be important for discriminating between chitotriose and cellotriose. Only clone 1 peptide indicated a specific binding to chitin-oligomer. This result suggests that a repetition of 'Thr/Ser-Arg-Thr/Ser' sequence would contribute for distinguishing methylcarbonylamino group of GlcNAc portion from hydroxyl group of glucose portion. In addition, the oxidized status, in which an SS linkage is formed should be required for the specific molecular recognition of chitotriose.

To date, several studies have reported chitin binding proteins. The binders obtained are listed as the following; a chitin specific lectin [11], an anti-chitin antibody [12], chitinase [13], etc. All the reported chitin binders have a chitin binding domains'. The length of the 'chitin binding domain' should be around 50 residues in amino acids. The dissociation constant between the present obtained peptide and chitotriose is not particularly low compared with other chitin

Table 3
Binding parameters of peptide clone 1 (oxidized) with chitotriose and cellotriose

Analyte	K_D (mol/l)	k_d (s^{-1})	k_a (l/(mol s))
Chitotriose	1.1×10^{-4}	1.7×10^{-4}	1.6
Cellotriose	1.3×10^{-1}	2.9×10^{-2}	2.2×10^{-1}

binders. However, the peptide obtained in this study would be the shortest chitin-binding unit. The usefulness of the new method of phage display screening using organic media [10] was demonstrated through this study.

3. Materials and methods

3.1. Screening of random peptide phage display library

In this study, we used pSKAN phage (MoBiTec, Göttingen, Germany) as library phages. Biopanning was performed according to our reported procedure [10]. In detail, 1.0×10^{10} pSKAN phages were subjected to a column of packed Chitooligo-agarose (Seikagaku Kogyo, Tokyo, Japan) buffered with washing buffer (50% EtOH in PBS (10 mM potassium phosphate buffer, 150 mM NaCl, pH 7.4), column void volume 0.2 ml) and incubated for 10 min at room temperature. The column was washed with 20 ml of the same washing buffer to remove the non-specific bound phage. The specific bound phage population was eluted with an elution buffer (50 mg/ml *N*-acetylglucosamine, in the washing buffer described above) and was reinfected with log-phase *E. coli* WK 6λ*mut*S and amplified for the next round according to the reported procedure [10]. After the five round repetition of the biopanning series, fifteen clones were randomly picked for DNA sequencing analysis by the dye terminator method using an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer Applied Biosystem Inc., Foster City, CA, USA).

3.2. Oxidation and reduction of peptides

Synthetic peptides (1 mg) were dissolved in 1 ml of hydrogen peroxide solution (1% in distilled water) and kept at room temperature for 10 min. The solution was lyophilized to yield oxidized peptides. Alternatively, synthetic peptides were dissolved in 2-mercaptoethanol solution (1% in distilled water) and kept at room temperature for 10 min. The solution was lyophilized to yield reduced peptides.

3.3. Binding assay of synthetic peptides for chitin

The binding assay with an SPR sensor (SPR 670, Nippon Laser and Electronics, Nagoya, Japan) was performed to detect affinity of the synthetic peptides for chitin-oligomer according to the our reported protocol [10]. We explain the assay system briefly as follows: A sensor chip coated with gold film (50 nm thick) (Nippon Laser & Electronics, Nagoya, Japan) was cleaned with ozone. The sensor chip was exposed to 10 ml EtOH containing 10 μM 4,4-dithiodibutyric acid for 30 min at room temperature. After washing with EtOH, the sensor chip was immersed in 10 ml of 90% dioxane containing 1-ethyl-3-(3-dimethylaminoproyl)-carbodiimide hydrochloride (EDC: 25 mg) and *N*-hydroxysuccinimide (NHS: 15 mg) with gentle shaking for 10 min. Subsequently, 10 ml pure water was added and the sensor chip was shaken gently for 5 min. Washing with pure water was repeated twice. The activated sensor chip was installed in an SPR apparatus (SPR670, Nippon Laser & Electronics, Nagoya, Japan). This SPR apparatus had a sensing and a reference cells. Fifty percent ethanol in PBS(10 mM potassium phosphate buffer, 150 mM NaCl, pH 7.4) was used as a running buffer at a flow rate 20 μl/min at 25 °C. Two 60 μl samples of synthetic peptides in distilled water (200 μg/ml) were independently injected into the sensing channel and the reference channel. Glycine solution (0.2 mol/l in distilled water) was used twice for blocking. The chitotriose solution (10 mmol/l in running buffer) was then injected into the sensing channel. After 300 s the running buffer was returned to the washing buffer without chitotriose and the signal value (angle) was observed. A subtraction of the signal value (angle) from the base line before the injection reveals the interaction between the immobilized synthetic peptides and chitotriose. At the same time, 10 mM cellotriose in the washing buffer was run into the reference channel as a control and the signal value was observed in the similar manner to evaluate binding specificity of the immobilized synthetic peptides. If synthetic peptides recognize chitotriose more preferentially, a subtraction value in the case when chitotoriose is the analyte should be greater than in the case of cellotirose. The detailed experimental procedure is referred to in our previous paper [10].

3.4. Kinetic analysis of binding affinity

An activated sensor chip was installed in the SPR sensor. Fifty percent ethanol in PBS (10 mM potassium phosphate buffer, 150 mM NaCl, pH 7.4) was used as a running buffer at a flow rate 20 μl/min at 25 °C. Sixty microliters of synthetic peptide solution (200 μg/ml in distilled water) was injected into only the sensing channel for immobilization onto the chip. After blocking with glycine solution (0.2 mol/l in distilled water), a series of cellotriose solutions (0.5, 2.0, 3.0, 4.0, 5.0, and 6.0 mmol/l) was sequentially injected into both the sensing channel and the reference channel simultaneously to evaluate the binding affinity. Glycine–HCl solution (10 mmol/l, pH 2.0) was injected in the interval between each concentration to regenerate the sensor chip. The difference between the sensing channel value and the reference channel value were subjected to a global fitting analysis for the kinetic study. A second similar experiment was performed according to the procedure described above except using chitotoriose instead of cellotriose.

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